Elongated Conformers of Charge States +11 to +15 of Bovine Ubiquitin Studied Using ESI-FAIMS-MS

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Recent advancements in high-field asymmetric waveform ion mobility spectrometry (FAIMS) have led to significant improvements in the application of this technology to the study of protein conformers. Compared with previous work, the maximum value of the separation voltage (i.e., the dispersion voltage) has increased, thereby enabling multiple, elongated conformers of individual charge states of bovine ubiquitin to be separated in the gas phase (e.g., four conformers of each of the +11 and +12 charge states were separated). The use of a carrier gas mixture of 40% nitrogen and 60% helium changed the separation selectivity compared with pure nitrogen and enhanced the signal intensity, especially for the +14 and +15 charge states (the latter was not detected in a nitrogen carrier gas). Conformer cross sections were determined using the FAIMS/energy-loss method and found to be similar within a given charge state. The cross sections for conformers of charge states +13, +14, and +15 plateau at about 2000 $Å^2$ suggesting that the structure of bovine ubiquitin is essentially unfolded after the addition of the 13th proton. (J Am Soc Mass Spectrom 2001, 12, 894–901) © 2001 American Society for Mass Spectrometry

Ubiquitin is a small protein that plays a central role in intracellular proteolysis in eukaryotes [1]. The interest in the ubiquitin-dependent proteolytic pathway is evidenced by a large number of reviews discussing this subject [2–7]. Investigating the structural chemistry of ubiquitin is important for learning more about its vital role in cellular metabolism [1]. Intact gas-phase ions of large molecules can be generated using electrospray ionization (ESI) [8, 9] and matrix-assisted laser desorption ionization [10], thereby allowing gas-phase studies to be carried out. These studies provide important complementary information to solution-phase studies, such as the role of the solvent in protein conformations [11].

Gas-phase conformational studies of bovine ubiquitin (76 amino acids, MW = 8565 Da) have been carried out using drift tube ion mobility spectrometry (DTMS) [12, 13] and hydrogen/deuterium (H/D) exchange experiments [14–16]. DTMS [17] separates protein conformers with different collision cross sections and has been used to separate compact, elongated, and partially folded conformers of bovine ubiquitin [12, 13]. Limited structural information relating to the elongated conformations was reported in these studies presumably because of the inability of DTMS to separate conformers with similar cross sections. In gas-phase H/D exchange [18, 19] the electrosprayed protein reacts with D_2O resulting in the displacement of some of the protein hydrogen atoms by deuterium atoms. The different levels of exchange are used to distinguish conformers, although at present, the relationship between exchange level and conformer shape is not well understood [20]. Nonetheless, H/D exchange studies with bovine ubiquitin have provided complementary information to DTMS by resolving conformers with similar cross sections. For example, H/D exchange was able to distinguish between two conformers of the +12 charge state [15, 16].

Conformers of bovine ubiquitin have also been investigated in the gas-phase at atmospheric pressure and room temperature using high-field asymmetric waveform ion mobility spectrometry (FAIMS) [21, 22]. The mechanism by which FAIMS distinguishes conformers is different from that of either DTMS or H/D exchange. The FAIMS device separates ions based on differences in their high-field (K_h) to low-field (K) mobilities (i.e., K_h/K) [23–25]. These differences are observed experimentally as the compensation voltage (CV) at which an ion is transmitted through a FAIMS device. In an earlier study [21], several experiments were carried out to show that multiple peaks within individual charge

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states in a CV spectrum were the result of different conformations of bovine ubiquitin. The continuous separation capabilities of FAIMS, which allow selected conformers to be introduced to the mass spectrometer indefinitely, were utilized in a second study [22] in combination with an energy-loss method [26, 27] for measuring collision cross sections. This study [22] showed that cross sections calculated using the FAIMS/energy-loss method for conformers of bovine ubiquitin were similar to those obtained by DTMS [13]. Furthermore, conformers with similar cross sections (e.g., two for the +12 charge state) that were not separated by DTMS, were shown to be separated by FAIMS.

Recent improvements to the FAIMS hardware have enabled the application of a higher dispersion voltage (DV) than was previously attainable. Increases in DV have previously been shown to increase both the CV of transmission and the signal intensity of many ions [24]. Increases in the CV of transmission are especially important to the analysis of conformers since, in general, these increases lead to a greater separation between different species [24]. The FAIMS carrier gas composition was also shown to be a critical parameter when optimizing the performance of a FAIMS device for analytical applications [28–31]. For example, a mixture of helium and nitrogen gas was shown to significantly increase both the CV of transmission and the signal intensity for morphine and codeine [31] compared with pure nitrogen gas. The effect of carrier gas composition on conformer separation has not been explored as previous studies of bovine ubiquitin using ESI-FAIMS-MS have employed only nitrogen as the carrier gas [21, 22]. The application of these improvements in FAIMS technology to the study of bovine ubiquitin conformers is described in this report. Elongated conformers of the high charge states (i.e., +11 and greater) are the focus of this study since the separation of these conformers using DTMS and gas-phase H/D exchange has had only limited success.

Experimental

Instrumental

The ESI-FAIMS-MS instrument used in this study has been described previously [22]. In brief, gas-phase ions of bovine ubiquitin were produced using ESI (4400 V, ~180 nA) and passed through a desolvation region before entering the FAIMS device. The curtain gas consisted of a mixture of nitrogen and helium gas (Air Products, Nepean, ON) with the percentage of helium being varied from 0 to 60%. These gases were passed through charcoal/molecular sieve filters separately and were then mixed together in a tee assembly. The total flow rate for all experiments was kept constant at 2.5 L/min. The gas flows were adjusted using mass flow meters (MKS Type 179A) that were controlled using an MKS Type 1179A mass-flow controller (MKS Instruments, Andover, MA). A portion of the curtain gas carried the ions through the FAIMS device (i.e., carrier gas) at a flow rate of ~0.5 L/min. The remainder of the gas flowed out of the desolvation region towards the ESI needle (i.e., desolvation gas).

An asymmetric waveform generator (750 kHz) capable of providing a dispersion voltage (i.e., the maximum peak voltage, DV) of up to −4400 V was used in this study. Ions transmitted by the FAIMS device were detected with a PE Sciex API 300 triple quadrupole mass spectrometer. By monitoring the *m/z* of a given ion while scanning the CV, an ion-selective (IS)-CV spectrum was generated. Mass spectra were collected at each of the peak maxima in the IS-CV spectra of the individual charge states.

Cross Section Measurements

Cross sections of bovine ubiquitin conformers were calculated using a procedure described previously [22]. Conformers separated by the FAIMS device were sampled by the mass spectrometer and introduced into the second quadrupole with well-defined initial energies (i.e., $E_0 = 10z$ eV, where z is the charge state). Nitrogen gas (UHP) was added to the second quadrupole via a leak valve, resulting in collisions between the protein ion (mass, m_1) and the neutral gas molecules (mass, m_2). These collisions caused the protein ion to lose some of its initial energy (E_0). The energy of the protein ion exiting the cell (E) was determined by monitoring the intensity of the ion beam while increasing the offset of the third quadrupole (i.e., stopping curve). The cross section for an ion (σ) could then be calculated from the equation:

$$\frac{E}{E_0} = \exp\left(-\frac{C_D n l m_2 \sigma}{m_1}\right) \quad (1)$$

Here, C_D is the drag coefficient for diffuse scattering (the calculation of which has been described elsewhere [27, 32]), n is the gas number density in the collision cell, and l is the collision cell length. Note that in this study, the pressure in the collision cell could not be measured directly. Consequently, the product nl was determined from an ion whose cross section was reported in the literature. A value $\sigma = 1970$ Å^2 for the +13 charge state of bovine ubiquitin was reported using DTMS [13]. For experimental conditions in FAIMS where only one peak is observed in the CV spectrum for the +13 charge state, the calibration of nl is straightforward. However, under certain experimental conditions, two conformers of the +13 charge state are separated (see Results and Discussion) with the signal intensity of one of these conformers being about 100 times greater than the other. For these conditions, the contribution of the minor conformer is assumed to be negligible and the calibration is based on the dominant conformer having $\sigma = 1970$ Å^2.

The reported cross sections were determined from the average of six cross section measurements. Two sets

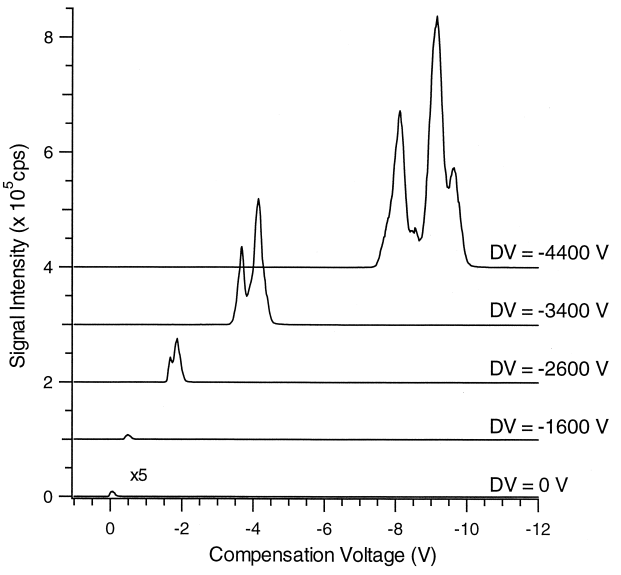

Figure 1. Ion-selective compensation voltage (IS-CV) spectra of the +12 charge state of bovine ubiquitin (*m/z* 714.8), collected at five dispersion voltages (DV), using a nitrogen carrier gas. The trace at DV = 0 V has been expanded vertically five times.

of three measurements for each conformer were acquired two weeks apart. The reported standard deviations represent the precision of all six measurements.

Chemicals and Solutions:

Bovine ubiquitin (90% purity) was purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. Solutions of 5 μM bovine ubiquitin in a solvent of 49/50/1 water/methanol/acetic acid (v/v/v) were used. When electrosprayed, this solvent composition favored the formation of higher charge states of bovine ubiquitin [22].

Results and Discussion

Effect of Dispersion Voltage on Conformer Separation

Recent improvements to the FAIMS asymmetric waveform generator have increased the maximum value of the dispersion voltage (DV). The voltages applied to the FAIMS device by the waveform generator (DV and CV) control the electric fields in the separation region. The effects of increasing DV on the CV spectra are illustrated in Figure 1 for the +12 charge state of bovine ubiquitin using a carrier gas of pure nitrogen. This figure shows a series of ion-selective compensation voltage (IS-CV) spectra obtained by monitoring the *m/z* of the +12 charge state (*m/z* 714.8) while scanning the CV from 1 to −12 V. The bottom trace in Figure 1 (expanded vertically 5 times), acquired using a DV of zero volts, shows a single peak centred at a CV of approximately zero. The low intensity (~1700 cps) is a result of ion loss to the walls of the FAIMS device via diffusion and space charge ion–ion repulsion. When a DV of −1600 V is applied, the peak for the +12 charge state appears at a CV of ~−0.5 V. This non-zero location of CV indicates that the electric fields between the cylinders are strong enough to generate differences between the high-field and low-field mobilities of these ions (i.e., $K_h/K \neq 1$). At a DV of −2600 V, the difference in ion mobilities is greater, resulting in a further increase in the CV of transmission. In addition, differences in K_h/K between species have increased [24, 33], thereby resulting in the detection of at least two distinguishable peaks in the CV spectrum. The trace at DV = −3400 V shows improved separation of these two peaks compared with the trace acquired at DV = −2600 V. A DV of −3400 V is the maximum voltage that was available in a previous study [22]. The top trace in Figure 1 was acquired at the maximum voltage (i.e., DV = −4400 V) supplied by the waveform generator used in this study. In this trace, four distinct peaks are observed for the +12 charge state. As was done in previous conformational studies of bovine ubiquitin using FAIMS [21, 22], a series of experiments were carried out at the CV values of each of the peaks in the CV spectrum for all of the charge states. These experiments showed that each of the four peaks for the +12 charge state was attributable to (at least) one different conformer. Note that the separation in the CV spectra shown in Figure 1 continues to improve up to the DV limit, suggesting that further increases in DV may lead to the separation of additional conformers.

In addition to improving the separation capabilities, increases in DV also have a significant effect on the signal intensity. Figure 1 shows that the maximum intensity has increased by over two orders of magnitude when comparing DV = −4400 V (~440 000 cps) with DV = 0 V (1700 cps). The increased sensitivity is due to an atmospheric pressure ion focusing mechanism. The strength of the focusing, and hence the signal intensity, generally increases as the CV increases [33].

Increases in DV will also cause additional heating of a conformer because of increases in its velocity as it moves through the bath gas. Based on calculations of the average effective temperature at high DV values, shown later in this document, the effect of the DV on the conformation is expected to be minimal.

Effect of Carrier Gas Composition on Conformer Separation

Carrier gas compositions other than pure nitrogen were investigated to determine if the separation of the bovine ubiquitin conformers could be altered. Of the small number of gas combinations that were investigated, the use of a gas mixture of 60% helium and 40% nitrogen resulted in the most significant changes compared with pure nitrogen gas. A helium content of greater than 60% resulted in electrical discharges in the FAIMS device at a DV of −4400 V and therefore was not used.

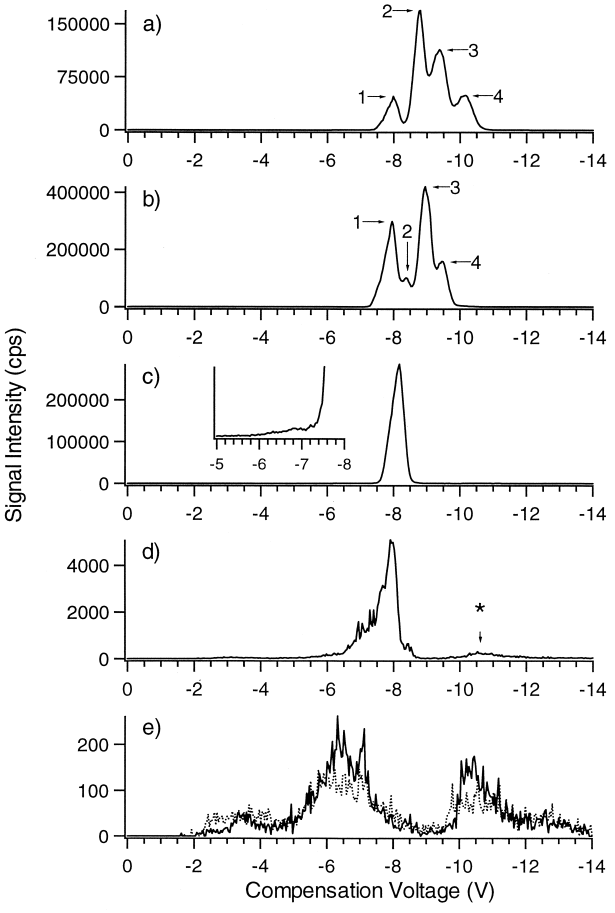

Figure 2. IS-CV spectra of bovine ubiquitin charge states: (**a**) +11 (m/z = 779.6), (**b**) +12 (m/z = 714.8), (**c**) +13 (m/z = 659.8), (**d**) +14 (m/z = 612.8), and (**e**) +15 (m/z = 572.0) using a nitrogen carrier gas and a DV of −4400 V. The inset in (**c**) shows a 100-fold expansion of the baseline. The peak labelled * in (**d**) is comprised of background ions with m/z 612.8. The dashed line in (**e**) represents the transmission of a background ion with m/z = 571.0.

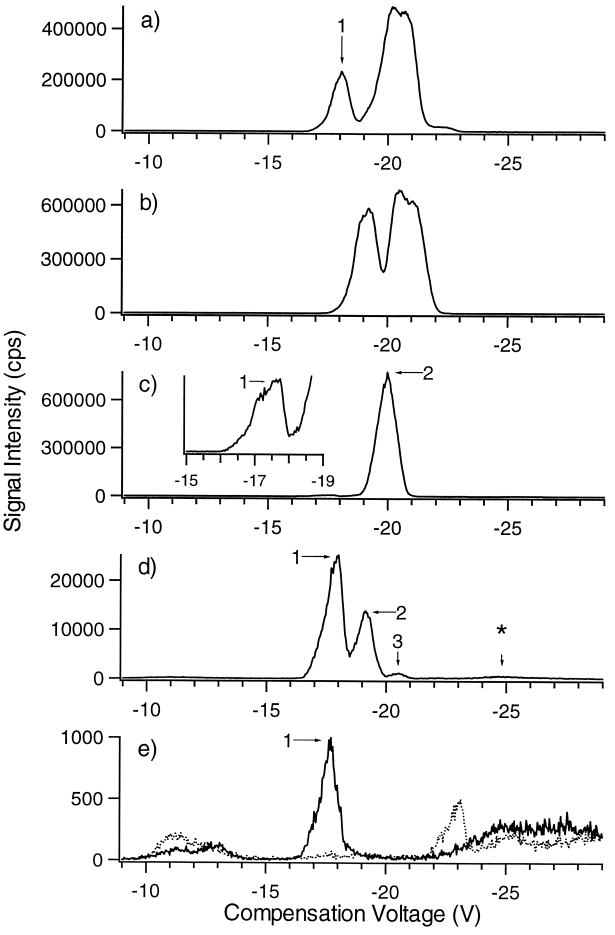

Figure 3. IS-CV spectra of bovine ubiquitin charge states: (**a**) +11 (m/z = 779.6), (**b**) +12 (m/z = 714.8), (**c**) +13 (m/z = 659.8), (**d**) +14 (m/z = 612.8), and (**e**) +15 (m/z = 572.0) using a carrier gas of 40% nitrogen and 60% helium, and a DV of −4400 V. The inset in (**c**) shows a 100-fold expansion of the baseline. The peak labelled * in (**d**) is comprised of background ions with m/z 612.8. The dashed line in (**e**) represents the transmission of a background ion with m/z = 571.0.

Figures 2 and 3 show IS-CV spectra for charge states +11 to +15 of bovine ubiquitin that were obtained under identical conditions with the exception of the carrier gas composition. Figure 2a–e were acquired using a carrier gas of 100% nitrogen, whereas Figure 3a–e were obtained using a carrier gas of 40% nitrogen and 60% helium. Note that additional CV spectra were also acquired by adding helium to the nitrogen carrier gas in 5% increments from 0 to 60% (not shown). These spectra were used to help interpret the differences between the spectra in Figures 2 and 3.

Figures 2a and 3a show IS-CV spectra for the +11 charge state of bovine ubiquitin obtained using ESI-FAIMS-MS at a DV of −4400 V. Figure 2a shows four peaks that were separated using a nitrogen carrier gas, each peak representing a different conformer. For ease in reference to a conformer, the notation cz_i will be used (z represents the charge state and i represents the peak number). For example, c11_1 refers to the conformer of the +11 charge state transmitted through FAIMS at the CV indicated by label 1. The same experiment acquired using a carrier gas of 40% nitrogen and 60% helium (Figure 3a) shows two major peaks in the spectrum at CV values of ~−18 V and −20.5 V, and a minor peak at a CV of −22.5 V. The peak at CV −20.5 V consists of both c11_2 and c11_3, which are overlapping in the CV spectrum. Thus, this peak is not labelled in Figure 3a since it is known to consist of multiple conformers that can be separated using a carrier gas of nitrogen. The minor peak in Figure 3a is also not labelled since c11_4 is not as well separated from c11_2 and c11_3 in 40% nitrogen and 60% helium as it was in 100% nitrogen. Similarly, other peaks that consisted of multiple species in one carrier gas that were separated in the other carrier gas are not labelled in Figures 2 and 3.

IS-CV spectra for the +12 charge state of bovine ubiquitin are shown in Figures 2b and 3b. At this DV, as was described in Figure 1, four conformers can be separated in a nitrogen carrier gas (Figure 2b). The use

of a carrier gas of 40% nitrogen and 60% helium (Figure 3b) shows only two peaks. Conformers c12_1 and c12_2 overlap in the CV spectrum at a CV of ~−19 V. Conformers c12_3 and c12_4 have also merged to give one broad peak at a CV of ~−21 V.

Figures 2c and 3c show IS-CV spectra for the +13 charge state. These spectra appear to be similar, as both are dominated by one intense peak. However, a 100-fold vertical expansion of the baseline to the left of the intense peak in each spectrum (shown in the inset) reveals a difference. The use of a carrier gas of 40% nitrogen and 60% helium has enabled the detection of a minor conformer (i.e., c13_1) that was not detected in nitrogen alone. Figures 2d and 3d show IS-CV spectra for the +14 charge state of bovine ubiquitin, which have much lower intensities than the IS-CV spectra for the +11, +12, and +13 charge states. The separation capability has dramatically improved in the carrier gas of 40% nitrogen and 60% helium compared with nitrogen alone (i.e., three peaks for the +14 charge state in Figure 3d compared with only one peak in Figure 2d). Note that mass spectra acquired at the CV corresponding to the maximum intensity for each of the peaks labelled by * did not show the presence of the +14 charge state. Instead, these peaks represented the location for transmission of background ions in the region near *m/z* 612.8.

Figure 2e shows an IS-CV plot of the *m/z* of the +15 charge state (i.e., *m/z* = 572.0) acquired using a carrier gas of nitrogen. The dashed line in this figure is a trace for background ions at *m/z* 571.0. The +15 charge state can not be distinguished from the background using a carrier gas of 100% nitrogen. Figure 3e shows an IS-CV spectrum acquired using a carrier gas of 40% nitrogen and 60% helium. A peak for the +15 charge state can be distinguished from the chemical background using this carrier gas composition. Figure 4, a mass spectrum (*m/z* 550 to 750) acquired at CV = −17.5 V using the same conditions as Figure 3e, confirms that the +15 charge state is present. The FAIMS device has filtered out much of the background noise at this CV, and charge states +12 through +15 are observed in Figure 4. In particular, the $[M + 15H]^{15+}$ and the $[M + 14H + Na]^{15+}$ species of bovine ubiquitin are readily distinguishable from the background at *m/z* 572.0 and *m/z* 573.4, respectively.

Although the focus of this work relates to the separation capabilities of FAIMS, the use of a 40% nitrogen and 60% helium carrier gas mixture instead of 100% nitrogen resulted in two other significant changes. The magnitude of the CV where the conformers are transmitted through the FAIMS device has increased and the absolute signal intensities for all of the individual charge states have increased. Note that the relative improvement in signal intensity appears to be dependent on the charge state since the most dramatic increases were observed for the +14 and +15 charge states.

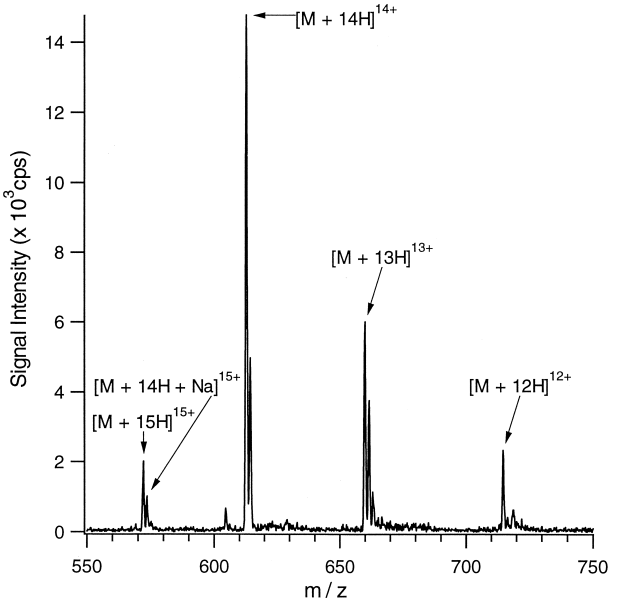

Figure 4. Mass spectrum from *m/z* 550 to 750 acquired at CV = −17.5 V using a carrier gas of 40% nitrogen and 60% helium, and DV = −4400 V.

Cross Section Measurements

Using an energy-loss method pioneered by Covey and Douglas [26], cross sections can be measured for conformers separated by the FAIMS device [22]. Table 1 gives a summary of the values for the cross sections, calculated using the FAIMS/energy-loss method, for all of the conformers identified in Figures 2 and 3. For conformers that were not separated using a particular gas mixture, no value was given. Only conformer c11_1

Table 1. Cross sectional areas for conformers of bovine ubiquitin separated at DV = −4400 V

Charge State	Conformer	Cross Section (Å²) Nitrogen only[a]	Cross Section (Å²) 40:60 Nitrogen/Helium[b]
15	c15_1	nd[c]	1993 ± 27
14	c14_1	ns[d]	2005 ± 15
	c14_2	ns	2002 ± 12
	c14_3	ns	2020 ± 26
13	c13_1	ns	1942 ± 10
	c13_2	ns	1970 ± 10
12	c12_1	1900 ± 14	ns
	c12_2	1929 ± 14	ns
	c12_3	1888 ± 11	ns
	c12_4	1875 ± 10	ns
11	c11_1	1849 ± 11	1842 ± 10
	c11_2	1829 ± 10	ns
	c11_3	1814 ± 11	ns
	c11_4	1798 ± 10	ns

[a] Based on the single observed peak of +13 having σ = 1970 Å² [13].
[b] Based on major conformer of +13 having σ = 1970 Å² (see experimental section).
[c] nd, conformer was not detected.
[d] ns, conformer was not separated from other conformers of the same charge state.

was observed not to separate into additional conformers or merge with other conformers when the carrier gas was changed from 100% nitrogen to 40% nitrogen and 60% helium. The cross section value for this conformer in 100% nitrogen (1849 ± 11 Å^2) agrees within experimental error with the value obtained using 40% nitrogen and 60% helium (1842 ± 10 Å^2).

Experimental Value for Near-Linear Conformation

Table 1 shows that the cross sections calculated using the FAIMS/energy-loss method plateau at ~2000 Å^2 after the addition of 13 protons to bovine ubiquitin. This plateau enables an estimate, based on experimental data, to be made for the cross section of the near-linear conformation. The experimental value of ~2000 Å^2 is somewhat lower than a reported value of ~2140 Å^2 for the calculated cross section of a near-linear conformer of bovine ubiquitin [12]. To our knowledge, conformers of the +14 and +15 charge states of bovine ubiquitin have not been reported using either DTMS or H/D exchange.

Influence of DV on Conformation

The influence of the experimental apparatus on protein conformation in the gas-phase is an important consideration. For example, in DTMS, the observed protein ion cross sections can be affected by the injection energy into the drift tube [12, 34]. When using a FAIMS device, the influence of the high electric field on conformation must be considered. Increases in the electric fields in the FAIMS device cause heating of a conformer because of increases in its velocity as it moves through the bath gas. The two-temperature kinetic theory of gaseous ion transport [35, 36] uses the effective temperature, T_{eff}, to characterize the relative kinetic energy in the ion-neutral center-of-mass frame. In particular, this theory gives an expression for T_{eff} as follows:

$$T_{eff} = T\left[1 + \frac{Mv_d^2}{3RT}(1 + \tilde{\beta}(T_{eff}))\right] \quad (2)$$

where R is the ideal gas constant, T is the gas temperature, M is the molar mass of the gas, $\tilde{\beta}$ is a small correction term (assumed to be 0 for these calculations), and v_d, the velocity of the ion, is given by:

$$v_d = N_0 K_0 (E/N) \quad (3)$$

Here, N_o is Loschmidt's constant (2.687×10^{25} m^{-3}), K_0 is the reduced mobility, E is the electric field, and N is the number density of the bath gas at ambient conditions.

During each cycle of the asymmetric waveform, E, and consequently v_d and T_{eff}, change as a function of time. Therefore, v_d and T_{eff} may be better represented as the average velocity ($\bar{v}_d$) and average effective temperature ($\bar{T}_{eff}$). To determine $\bar{v}_d$, the magnitude of v_d values calculated during one complete cycle of the asymmetric waveform are averaged. With this value, $\bar{T}_{eff}$ can be calculated in a way analogous to T_{eff} in eq 2 using $\bar{v}_d$ instead of v_d. Note that K_0, which cannot be determined from data obtained with the FAIMS device, was assigned an average value of ~0.7 cm^{2}V^{-1}s^{-1} based on a literature value for the +5 through +11 charge states of bovine ubiquitin [37]. Slight variations from this value, due to the mobilities of the individual charge states, will have minimal effect on these calculations. Table 2 summarizes the influence of the DV on $\bar{T}_{eff}$ for bovine ubiquitin ions over the DV range used in acquiring the data for Figure 1. The table shows that $\bar{T}_{eff}$ changes by less than 7 K over the range of DV used in this experiment. The maximum T_{eff}, also given in Table 2, represents the instantaneous value at the maximum voltage applied by the asymmetric waveform. Note that the ion only experiences these extreme values of T_{eff} for short periods during each cycle of the waveform. For example, during the application of DV = −4400 V (waveform frequency of 750 kHz), the calculated instantaneous value for T_{eff} exceeds 305 K (max 327.2 K), for only ~23% of the time. Based on these calculated values, changes to the 3-dimensional structure of the conformers, due to heating of the ions by the strong electric fields, are expected to be minimal.

Table 2. Influence of DV on the maximum effective temperature and the average effective temperature for bovine ubiquitin ions with K_0 of 0.7 cm^{2}V^{-1}s^{-1} [37].

DV	max T_{eff}(K)	$\bar{T}_{eff}$
0	296.0	296.0
−1600	300.1	296.9
−2600	306.9	298.4
−3400	314.6	300.1
−4400	327.2	302.8

Conformer Separation in FAIMS

Table 1 gives the cross sections calculated using the FAIMS/energy-loss method for the conformers of charge states +11 through +15. The cross section values are very similar within a given charge state indicating that properties other than cross section dictate the optimum CV of transmission of a conformer through the FAIMS device. As discussed in the Introduction, both DTMS and H/D exchange experiments have also been used to study the conformers of bovine ubiquitin. Only single peaks were observed for the +11 to +13 charge states with denaturing solutions using DTMS [13]. H/D exchange identified two conformers for each of the +11 [15] and +12 charge states [15, 16], but only one conformer for the +13 charge state [15]. Freitas et al. [15] suggested that subtle differences in the location of the charge do not significantly change the cross section but can lead to large differences in exchange rates.

At present, the properties of a conformer that dictate its location in a CV spectrum are largely unknown. The results presented herein show that the FAIMS device is able to respond to different structures of the conformers of the high charge states that are only slightly (or not at all) different in cross section. These ions may differ in their 3-dimensional structures or the FAIMS device may be responding to differences in charge state location along the protein ion. Note that, as was stated by Cassady and Carr [16], these two options should not be considered as mutually exclusive. Bovine ubiquitin contains 13 basic sites (7 lysines, 4 arginines, 1 histidine, and the N-terminal amino group). A large difference exists between the gas-phase basicities for the remaining amino acids in bovine ubiquitin compared with the gas-phase basicities for the basic sites [38]. If intramolecular effects are ignored, based on an argument of charge location, the dominance of one conformer for the +13 charge state might be expected since all of the basic sites on this charge state will be preferentially protonated. Multiple conformers for the +11 and +12 charge states may be expected since there are several possible ways to arrange these charges on the 13 basic sites. In addition, for charge states higher than +13, multiple conformers may also be expected because the differences in gas-phase basicities between the remaining amino acids are relatively small and the additional proton may be located at several sites. Although the spectra in Figures 2 and 3 are consistent with arguments of charge location, a more detailed investigation is required. For example, at higher DV values, or with the use of a different carrier gas composition c13_2 may be separated into multiple conformers. Studies involving the investigation of simpler ions, such as small peptides, are ongoing and may help to ascertain if charge location is an important parameter in FAIMS.

Charge Stripping Reactions

A proton stripping reaction in the interface of the mass spectrometer might add complexity to the CV spectra by producing phantom peaks. The possibility of charge stripping reactions has been considered and is discussed using the following examples. Several ions of bovine ubiquitin appear in the mass spectrum shown in Figure 4. This mass spectrum (*m/z* 550 to 750) was collected at CV = −17.5 V, and is therefore expected to include all ions that are transmitted through FAIMS at this particular CV. If charge stripping was occurring in the interface, a scan of CV would reveal a perfect overlap of sequential charge states, since the secondary products are required to appear at the same CV as the primary ion. For example, proton stripping of the most intense conformer of the +14 charge state, c14_1, would be expected to yield a +13 peak with the *same* shape over the *same* CV range. Figure 5a shows the IS-CV plots for charge states +13 and +14 over a narrow CV range in which the intensities of the conformers c14_1 and c13_1 have both been normalized to 1.0. This figure shows that that the c14_1 is observed over a different CV range then either c13_1 or c13_2. Thus, this figure illustrates that neither c13_1 or c13_2 can be a stripping product of c14_1. Figure 5b shows a similar plot for the +14 and +15 charge states (normalized to the conformers c14_1 and c15_1). Although these conformers are not as well separated as the example shown in Figure 5a, there is still a significant difference in CV and peak shape that shows that c14_1 cannot be a stripping product of c15_1.

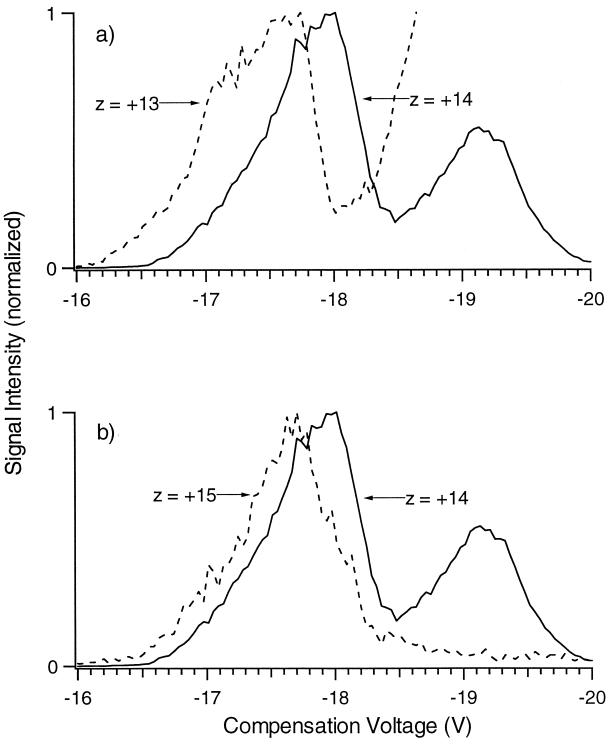

Figure 5. IS-CV plots of (a) the +13 and +14 charge states normalized to conformers c13_1 and c14_1, and (b) the +14 and +15 charge states normalized to conformers c14_1 and c15_1.

Conclusions

Conformers of highly charged gas-phase ions of bovine ubiquitin (i.e., +11 to +15) were examined using ESI-FAIMS-MS. Application of higher asymmetric waveform voltages (i.e., DV) than previously attainable are shown experimentally to improve the separation capability. The use of a carrier gas of 40% nitrogen and 60% helium was also shown to change the separation capabilities compared with 100% nitrogen. Based on these advancements, several conformers of bovine ubiquitin were detected that have not been previously identified by H/D exchange or DTMS. Cross sections for the conformers were calculated using an energy-loss technique and were found to be similar within a given charge state. A value of ~2000 Å^2 for the near-linear conformation was estimated based on these experimentally derived cross section values.

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